Identification of cost effective plantlet regeneration method for commercial-scale date palm micropropagation

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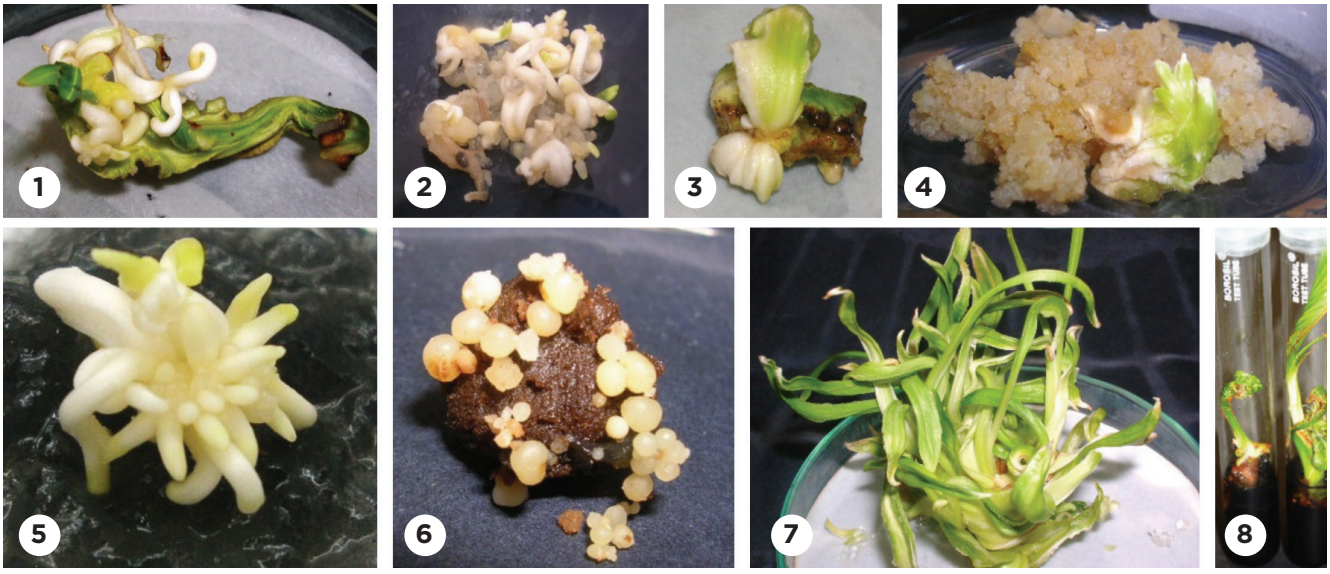
ABSTRACT

An investigation on multiple plantlet regeneration methods from date palm shoot tip and leaf explants was carried out at the Plant tissue culture unit of the Environment and Life Sciences Research Centre of Kuwait Institute for Scientific Research to identify the most cost effective method of plantlet regeneration for date palm micropropagation. Four cultivars namely Barhi, Madjhool, Khlas and Suckari were used for the experimental study. The media and protocol developed for the date palm micropropagation at KISR were used for the regeneration experiments. Eight different in vitro methods of plantlet regeneration were developed during the study. Each method had its own merits and demerits. Plantlets were produced through each method of in vitro regeneration method, hardened and planted in the field for evaluation. Plants produced through all the 8 different methods of regeneration were confirmed true-to-type in the field. Economic feasibility study was conducted for each method of regeneration. Among all the eight different methods of plant regeneration, direct somatic embryogenesis method of regeneration was identified as the most cost effective method of regeneration for the commercial-scale date palm micropropagation.

Keywords: Tissue culture, somatic embryogenesis, organogenesis, acclimatization, in vitro.

INTRODUCTION

Date palm (Phoenix dactylifera L.) is propagated traditionally by offshoots or suckers, which are produced in the leaf axils and usually appear at or below the ground level surrounding the stem base. Small offshoots that appear above the ground level on the trunk are usually destroyed due to difficulty in rooting. Offshoots are produced in a limited number for a certain period in the lifetime of a young date palm tree. Offshoot formation is dependent on the



Figures1-8. Date palm in vitro regeneration types. Fig. 1. Direct somatic embryogenesis; Fig. 2. Indirect somatic embryogenesis; Fig.3. Direct organogenesis; Fig. 4. Indirect organogenesis; Fig. 5. Somatic embryogenesis from shoot bud; Fig. 6. Meristemoids regeneration; Fig. 7. Axillary shoot induction; Fig. 8. Plant regeneration from shoot meristem.

genetic makeup of the cultivar and the environmental factors. The number of offshoots produced by an individual date palm tree is highly variable and varies cultivar to another. The traditional method of vegetative propagation through offshoot is slow, laborious, time-consuming and expensive. Transmission of disease-causing pathogens and insects is another disadvantage of conventional offshoot propagation. This has focused on micropropagation technology development during the past 40 years for rapid clonal reproduction of selected cultivars which is cheaper than the offshoot method.

Date palm tissue culture was initiated with little success in the year 1970 (Schroeder, 1970) and succeeded in developing protocols during late 1970s (Ammar and Benbadis, 1977; Tisserat, 1979). Free-living date palm plantlets using tissue culture method was succeeded during 1980s using shoot tip tissue explants (Tisserat and DeMason, 1980; Tisserat, 1981; Beauchesne, 1982; Zaid and Tisserat, 1983). The culture media and protocol was either adopted or modified and used for the micropropagation of date palm worldwide later on (Sharma et al, 1986; Sudhersan, 1989). Date palm micropropagation using immature flower buds was reported in 1985 (Drira and Benbadis, 1985). Several date palm cultivars have been micropropagated in various laboratories in different parts of the world. Organogenesis and somatic embryogenesis are the common methods of regeneration practiced for this purpose (Beauchesne, 1982; Reuveni, 1979; Sharma et al., 1986; Sudhersan et al., 1993). Literature study on date palm micropropagation indicated that complicated culture media with different combinations of growth hormones and organic additives were reported for plantlet regeneration. In the present study a simple and efficient protocol was established to regenerate plantlets through multiple regeneration methods. Generally occurring tissue browning, in vitro plantlet dwarfing and hyperhydricity were also controlled through this simple protocol. Plantlets produced by all this methods were proved to be clonal in nature (Sudhersan and AboEl-Nil. 2004). The details of the study are presented in this report.

MATERIALS AND METHODS

Plant Material

Offshoots of date palm cultivars Barhi, Khlas, Madjhool and Succary were used as plant material. All the offshoots were collected from the date palm orchard maintained inside the Kuwait Institute for Scientific Research (KISR) campus, Shuwaik, Kuwait.

Explant Preparation

The older leaves of the offshoots were dissected out acropetally and the shoot tip with few young leaf primordia were isolated and surface sterilized prior to explant preparation. Surface sterilization

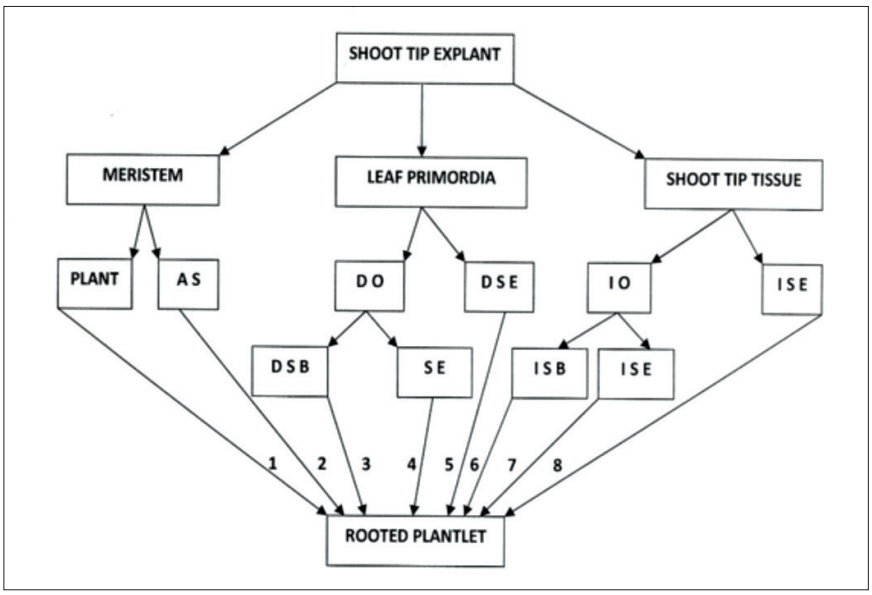


Fig. 9. Different regeneration methods of plantlet regeneration in date palm. AS-Axillary shoot; DO-Direct organogenesis; DSE-Direct somatic embryogenesis; IO-Indirect organogenesis; ISE-Indirect somatic embryogenesis; DSB-Direct shoot bud; SE-Somatic Embryogenesis; ISB-Indirect shoot bud.

was carried out using 100% commercial Chlorox with a drop of Tween 20 for 30 min followed by thorough washing in sterile distilled water for five to six times. All surface sterilization activities were carried out under the laminar hood. The explants were isolated using a sterile forceps and scalpel with surgical blade under aseptic conditions. The explants (whole leaf primordia with leaf sheath, shoot meristem with stem tissue and irregularly cut shoot tip tissue) were prepared for the regeneration experiments. Two offshoots from each cultivar were used for the study.

Culture Medium

The culture medium used by Tisseret (1979) was modified and formulated a five stage culture media: 1. initiation, 2. regeneration, 3. growth and multiplication, 4. elongation and rooting and 5. photoautotrophic culture for this study. The pH of the media was adjusted to 5.6 using 1 N NaOH or 1 N HCl before adding the agar. All media were sterilized by autoclaving at 121° C and 101Kpa for 15 min.

Culture Initiation and Incubation

Two sets of explants prepared from each cultivar were inoculated aseptically on to the initiation media. One set of cultures were maintained under total darkness at 25º C temperature and another set was maintained at 16 h light and 8 h dark culture condition. Cultures maintained at total darkness was subcultured in the same media once in 30 days and cultures maintained at light were subcultured once in a week. After 60 days all the explants were transferred to the regeneration media.

Regeneration

After transferring to the regeneration media, 50% of the cultures including the shoot meristem maintained at total darkness were transferred to 16 h light and 8 h dark culture condition and the remaining 50% were maintained at total darkness. All cultures were subcultured once in 15 days regularly in the same regeneration media. Observations were made regularly once per week on callusing and direct regenerations on the tissue explants. All types of regeneration methods were photographed and recorded.

Growth and Multiplication

Tissue explants or leaf primordial expalnts showing embryo or shoot bud were transferred to the growth and multiplication media and subcultured once in every 15 days regularly. Somatic embryos or shoot buds were isolated and maintained in the same media during each subculture. Germinated plantlets were isolated and subcultured in elongation and rooting media.

Elongation and Rooting

Germinated plantlets were isolated and transferred to the elongation and rooting media and maintained for 20-30 days. After 30 days all the rooted plantlets were removed for photoautotrophic culture and the remaining un-rooted plantlets were subcultured in the same rooting medium for rooting.

Photoautotrophic Culture

Photoautotrophic culture media was prepared by mixing quarter strength MS macro and micro nutrients (Murashige and Skoog, 1962) with a soil mix prepared by mixing peat moss and garden soil at 1: 1 ratio and autoclaved at 121° C for 30 min and cooled at room temperature. Rooted plantlets were washed in soap solution and rinsed in sterile water for three times and planted in plastic trays containing 100 planting cells of

5cm side and 10cm height. All plantlets were sprayed with 0.5 g/l solution of fungicide Topsin® and kept inside the poly carbonate transparent culture box in closed condition. Photoautotrophic culture boxes were maintained inside the growth rooms at 25±2˚C, 3000 lux light intensity and 12 h photoperiod. After 30 days all boxes were transferred to the greenhouse for acclimatization.

Acclimatization

All the plantlets were maintained inside the temperature and humidity controlled greenhouse for a week time and the boxes were kept opened for 4 h daily for another week and the plantlets were exposed to the greenhouse conditions completely afterwards. All plantlets were transferred to same soil media in larger pots and maintained in the nursery benches for 30 days to grow and later on transferred to 1gallon plastic pots and maintained in the shade-house for field transfer.

RESULTS

Effect of light

The cultures maintained at total darkness did not show any browning. All cultures kept under light started browning. Browning of tissues maintained at light was controlled by subculture frequently at 7 d interval. Greening and tissue maturation were observed in expalnts maintained at light. All tissue explants turned to light yellowish in color under total dark condition. Callusing was delayed in light and callusing was faster under dark. Direct somatic embryos and shoot buds were regenerated faster under light and slow under total darkness. Percentage of regenerating tissue explants was more in dark and less under light.

Effect of Explants

Among the whole leaf primordia, shoot meristem and irregularly cut shoot tip tissue, irregularly cut tissues callused faster and produced callus within 60 days under total darkness. The whole leaf primordial explants produced callus only at the basal sheath region and at the laminal region. Shoot meristem enlarged and did not produce callus within 60 days period. Globular meristomoids were observed on the cut end of the tissues initially prior to callus initiation under dark condition. Under prolonged dark condition, the meristemoids turned in to callus in the initiation media. When transferred to the regeneration media and maintained at light, the meristomoids produced somatic embryos or shoot buds. The shoot meristem gradually turned into green and elongated into plantlet within 90 d. The irregularly cut tissue explants transferred in to the regeneration media and maintained under light produced somatic embryos and shoot buds directly or through callus. There were three types of calli: 1. mucilaginous, 2. nodular and 3. spongy observed on the cut tissue explants. The whole leaf explants showed direct regeneration.

Types of Regeneration

After 30 days under darkness in the initiation media, the explants prepared through irregular cuts showed induction of globular meristemoids. After another 60 days under the darkness in the same initiation media under total darkness, calli were proliferated and three types of calli were recognized: 1. nodular, 2. spongy and 3. mucilaginous. When transferred to the regeneration media under light, nodular callus differentiated into green organogenic and yellowish embryogenic callus. The mucilage callus produced only yellowish embryogenic callus. The whole leaf primordial explants produced meristomoids at the basal sheath area and laminal tissue area in the initiation media. When the leaf primordial explants transferred to the regeneration media and maintained under light, produced somatic embryos and shoot buds directly. The shoot meristem explant when transferred to the regeneration media and maintained at light turned in to green and elongated in to plantlet with compound leaf. Axillary shoots were appeared from the leaf axils. A total of 8 types of plantlet regeneration: 1. Direct somatic embryogenesis, 2. Indirect somatic embryogenesis, 3. Direct shoot induction, 4. Indirect shoot induction, 5. Axillary shoot induction, 6. Adventitious shoot formation from the shoot buds, 7. Direct somatic embryogenesis from shoot buds and 8. Direct plant regeneration from meristem (Figs. 1-9).

Plantlet Regeneration

Plantlets were produced in growth and multiplication media through all the 8 different methods. The somatic embryos multiplied by secondary somatic embryogenesis and germinated in to plantlets. Shoot buds produced plantlets directly or through somatic embryos produced at the basal region of the shoot buds. Shoot meristem produced axillary shoots and plantlet. More number of plantlets were produced through somatic embryos obtained through any method of regeneration. Plantlets were produced continuously using the multiplication and growth culture media.

Photoautotrophic Culture

In the photoautotrophic culture media, plantlets were more hardened and the roots were

multiplied. More capillary roots were also induced. The leaves turned dark green in colour. The surface of the leaves produced more waxy coating on the surface during the photoautotrophic culture phase.

Acclimatization

Plantlets produced through all the regeneration methods were hardened through two methods: 1. direct transfer from the rooting stage and 2. transfer from the photoautotrophic culture. Plantlet survival was 50% by the first method and 100% through the second method. Plantlets acclimatized through photoautotrophic method showed faster growth.

Field Evaluation

Plantlets produced through all the 8 types of regeneration method were planted in the date palm orchard inside the KISR campus and maintained. All the palms produced by all the eight different methods showed faster vegetative growth, produced axillary shoots during the vegetative growth phase and started producing flowers during the 4th year of planting. Palms produced by all the eight different methods were found to be true to type.

Feasibility

The minimum cost for producing 25,000 plants through each method was calculated. among all the eight methods studied, the direct somatic embryogenesis method was the most efficient and highly economically feasible method of plant production.

DISCUSSION

Date palm micropropagation research started during1970s (Hoded, 1977; Reuveni et al., 1974; Tisserat et al., 1979). Immature or mature somatic embryos were cultured on MS culture media containing many organic additives, an auxin (2,4-D) and a cytokinin (2iP) or kinetin (K) for callusing and somatic embryogenesis. During the latter stages, somatic embryogenic calli were obtained from tissue explants from the shoot tip and plantlets were regenerated and planted in the year 1982. Starting from 1982 many laboratories carried out research and development on date palm micropropagation. Literature study indicated different types of regenerations, complicated culture media formulations and physiological disorders like tissue browning, hyperhydricity and dwarfing which affected plant production (Zaid and De Wet, 2002). However, many laboratories developed their own techniques for the date palm micropropagation through somatic embryogenesis or organogenesis.

Through 15 years of research and development on date palm micropropagation, we have developed a five stages of culture media through modifications of the culture media reported by Tisseret (1979). Through this five different stages of culture media we were able to produce large number of plantlets without any genetic disorders. Techniques were also developed for controlling tissue browning and dwarfing of in vitro plantlets without any changes in media components. This media was successfully used for the micropropagation of 40 different date palm cultivars in our laboratory.

Generally according to the published reports, the shoot tip used to be cut irregularly into small pieces and used as explants for somatic embryogenesis, and leaf primordial explants were used for organogenesis. The initiation media used to have the growth hormone 2,4-D for somatic embryogenesis and auxins other than 2,4-D and cytokinins for organogenesis. Under total darkness the tissue explants produce callus after 3-6 months and later the nodular callus produces somatic embryos in the case of somatic embryogenesis method and shoots were inducted from the leaf base in the case of organogenesis. In our study, we have observed multiple regeneration method using a single culture initiation media through manipulation of explants and timing on transfer from initiation media to regeneration media.

Initially, tissue explants of date palm in the presence of 2,4-D produces globular structures called meristemoids at the cut ends of the tissue explants after 30 d of culture. On prolonged culturing in the same media, the meristemoids grow further and produces different types of callus. If the explants with meristemoids are transferred to the regeneration media and maintained under light, the meristemoids turned to develop into shoot bud or somatic embryos directly. From this it is clear that the growth hormone 2,4-D induces the regeneration potential of the explants and supported callusing afterwards. The leaf margins, leaf base, leaf sheath rachis and petioles are potential in regeneration.

The maintenance of the explants with 2,4-D induced meristemoids (proembryos) on the same media for 90 d induce somatic embryogenic callus. On transferring these somatic embryogenic callus to the maturation media produces somatic embryos followed by secondary somatic embryos. The direct somatic embryogenesis and indirect somatic embryogenesis occurred in the same culture medium and the culture time factor controls the two types of embryo production. Removal of

somatic proembryos from the 2,4-D medium at the right time and subculture to maturation media avoids the somatic embryogenic callus formation. Secondary somatic embryo formation was found to be a continuous process which occurred in PGR-free MS culture media for several years when the cultures were subcultured periodically once in 15 d. The mature somatic embryos germinated into plantlets with a single thin tap root. On trimming the taproot and transferring the plantlets to a rooting media containing produces several healthy adventitious roots after 20-30 d in culture. One hundred percent plantlets produced by our protocol survived during acclimatization. However, less than 5% plant mortality was observed during the long term nursery maintenance.

Our study concluded that the auxin 2,4-D is mainly necessary for any type of regeneration at the culture initiation stage. Timing and explant type are the important factors which decides the type of regeneration. Among all the 8 different types of regeneration methods, direct or indirect somatic embryogenesis is preferred to commercial production. All other methods can be used for the experimental purpose. Organogenesis is a slow method which takes 12 -24 months for the initial establishment. However, after producing the adventitious plantlet multiplication stage, plant production will be faster and similar to the somatic embryogenesis method. Through the rejuvenation method, very old trees of commercially important characters can be rejuvenated in to young plants. Our protocol works out for clonal propagation of any date palm cultivar. Among all the possible methods of plant production, direct somatic embryogenesis method was found to be highly economically feasible method.

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